Population Genetics of Long-Tongued Nectar Bat, *Macroglossus minimus* (Chiroptera: Pteropodidae) in Malaysian Borneo.

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This project is submitted in fulfilment for the requirement for the degree of Bachelor of Science

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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LIST OF ABBREVIATION

%	Percentage
°C	degree Celsius
µl	microliter
ca	centiare
cm	centimetre
CTAB	Cetyl- tri- methyl ammonium bromide
Cyt *b*	Cytochrome *b*
dNTPs	Deoxyribonucleotide triphosphates
EtOH	Ethanol
g	gram
m	metre
MEGA	Molecular Evolutionary Genetic Analysis
$MgCl_2$	Magnesium chloride
ml	millimetres
ML	Maximum Likelihood
MP	Maximum Parsimony
mtDNA	mitochondrial Deoxyribonucleic acid
PAUP	Phylogentic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
Taq	*Thermus aquaticus*

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ABSTRACT

The long-tongued nectar bat, *Macroglossus minimus* is the smallest of the family Pteropodidae commonly found both in primary and secondary tropical moist forest. *M. minimus* is chosen as a model for this study because of lack information on their genetic relationship within the species itself. The main objective of this study is to determine the genetics structure of *M. minimus* from different populations in Malaysian Borneo using partial primer of cytochrome *b* (cyt *b*) mitochondrial gene. This population is hypothesised to show genetic variation due to the isolation by geographical distance. The result showed that there are no genetic variations among *M. minimus* populations in Sarawak. However, there are genetic divergence between populations of Sabah and southern part of Sarawak. The variation of genetic structure might be related to historical event. More samples should be included in future studies to confirm the genetic divergence of the species within and among their populations.

Keywords: Genetic, *Macroglossus minimus*, partial cytochrome *b*, genetic divergence, Malaysian Borneo.

ABSTRAK

Kelawar pemakan madu berlidah panjang adalah yang terkecil dari famili Pteropodidae kebiasaanya dijumpai di hutan lembap tropika rendah dan tinggi. M. minimus dipilih menjadi model untuk kajian ini kerana kurangnya informasi berkaitan dengan hubungan genetik antara spesis itu sendiri. Objektif utama kajian ini dijalankan adalah untuk menentukan struktur genetik M. minimus dari beberapa populasi di Malaysia Borneo menggunakan sebahagian daripada jujukan cytochrome b (cyt b) mitokondria. Populasi ini dianggap mempunyai genetik variasi disebabkan oleh pengasingan jarak gegografi. Kajian mendapati bahawa tiada variasi genetik dalam populasi-populasi M. minimus di Sarawak. Walaubagaimanapun, terdapat genetik variasi di antara populasi M. minimus di Sabah dan selatan Sarawak. Variasi dalam genetik struktur ini berkemungkinan berkait rapat dengan peristiwa lampau. Lebih banyak sampel perlu dimasukkan dalam kajian seterusnya bagi memastikan perbezaan genetik di antara dan dalam populasi spesies tersebut.

Kata kekunci: Genetik, M. minimus, sebahagian jujukan cyt b, perbezaan genetik, Malaysia Borneo.

CHAPTER 1

INTRODUCTION

Bats are placed in Order Chiroptera. They are widely distributed and the second most diverse groups in the world after the rodents (Altringham, 1996). It is divided into two distinct suborders, namely, Megachiroptera and Microchiroptera, which comprises of 18 family and consisting approximately of 202 genera and 1116 species (Wilson and Reeder, 2005). Megachiroptera includes all frugivorous, nectarvorous, and folivorous bats, while Microchiroptera consists of insectivorous and carnivorous bats (Altringham, 1996; Mickleburgh *et al*., 1992; Alvarez *et al*., 1999).

Pteropodidae is the only family under suborder of Megachiroptera (Payne *et al.*, 1985). There are 42 genera and 166 species of Pteropodidae recorded worldwide. Pteropodids consist of all flying foxes and Old World fruit bats which are further divided into four subfamilies, namely, subfamily Pteropodinae, Harpyionycterinae, and Nyctimeninae (Corbet and Hill, 1992). The Malaysia pteropodids are consisting of subfamily Pteropodinae and subfamily Macroglossinae (Corbet and Hill, 1992).

Macroglossus minimus or long-tongued nectar bat are widely distributed species. They can be found from Borneo, Peninsular Malaysia, Philippines, Singapore, South Thailand and Vietnam (Mickleburgh *et al*., 1992). In Malaysia Borneo, it has been recorded from most areas including Kota Kinabalu, Witi Range, Sepilok, Sukau and Tawau in Sabah, and near Niah and Bako in Sarawak (Payne *et al.,* 1985). It is occasionally found roosting beneath the dead leaves of banana and usually only a single individual or a mother with a single young are found at the roost site (Mickleburgh *et al*., 1992). Foraging behaviour of *M. minimus* is similar to *Eonycteris spelaea* by landing on inflorescences and using its long tongue to pick up nectar and pollen, and to groom pollen from its fur (Start and Marshall, 1976).

Kofron (2008) have studied the reproduction of *M. minimus* in Brunei, and found that the female *M. minimus* in Brunei have a reproductive cycle that is consistent with a pattern of seasonal bimodal polyoestry. However, the knowledge on phylogeny of *M. minimus* is lacking especially their genetic structure from different populations in Malaysian Borneo as well as a whole Malaysia. The genetics information of the species is crucial for their future management and conservation. As Megachiropteran, *M. minimus* plays an important role in ecological maintenance as they act as seed dispersers and also as a pollinator for some fruits and flowers. The introduction of molecular techniques is a great breakthrough in the pursuit of the understanding of a species population structures. The use of genetic markers has led to the description and a better understanding on social life (Bryja *et al*., 2009).

The cyt *b* has been considered as one of most useful genes for the phylogenetic work and probably best known mitochondrial gene with respect to structure and function of its protein product (Irwin *et al*., 1991). The primer cyt *b* is chosen because it is easier to align a protein coding sequences that has evolved over the period spanning the origin of mammalian order and has been used for the diversity of systematic questions because it is sufficiently variable for population analysis and conservative enough for phylogenetic analysis (Campbell *et al*., 2004; Irwin *et al*., 1991).

1.1 Objective

The objective of this study is:

1. To study the genetic structures and variation among different populations of *M. minimus* in Malaysian Borneo using partial mtDNA cyt *b* gene.

1.2 Hypothesis

The hypothesis of this study is:

H_o: There is no variation in genetic structures among different populations of *M. minimus* in Malaysian Borneo.

H_A: There is variation in genetic structures among different populations of *M. minimus* in Malaysian Borneo.

CHAPTER 2

LITERATURE REVIEW

2.1 Population genetics

Numerous studies on population genetics of chiroptera were conducted in Indo-Malayan region. Abdullah (2003) studied on genetic variation of *Cynopterus brachyotis* of Southeast Asia. He found two different forms of *C. brachyotis* according to the habitat usage and supported by separated grouping within phylogeny tree suggesting the occurrence of cryptic in the putative species.

Rahman (2005) studied the phylogenetic relationships of Macroglossinae inferred by using the partial mitochondrial DNA cytochrome *b* gene. She detected a high genetic distance between *Eonycteris spelaea* and *E. major* (11.2-11.8%) suggested that these species are totally divergent from each other. Besides that, she also found that there are high genetic divergence (24.0-27.0%) between *M. minimus* and *M. sobrinus*.

According to Tingga and Abdullah (2012), there were two major clusters within the genus *Aethalops*. *A. aequalis* in Borneo were clearly distinguished from *A. alecto* from the islands of Indonesia. Meanwhile, Mohd Ridwan and Abdullah (2012) found that the populations of

Penthetor lucasi were separated into two haplogroups, namely, Haplogroup 1 (found in Miri and Kuching populations) and Haplogroup 2 (Miri, Kuching, Sri Aman and Kelantan populations). Other genetic analysis, based on partial cytochrome *b* gene, shows the high genetic variation within *Myotis muricola* and members of *M. muricola* Eastern are grouped together independently of *M. muricola* Western and both groups are distantly related (Wiantoro *et al*., 2012).

2.2 Study species

M. minimus has body length of approximately 60-85 mm long with the extremely short tail, reaching to 4 mm at the longest length. The fur of *M. minimus* is reddish-brown in colour on its back and is very long compared to other species of Pteropodidae. It is can be found in both primary and secondary tropical moist forest. They are distributed in Borneo, Peninsular Malaysia, Philippines, Singapore, South Thailand and Vietnam (Mickleburgh *et al*., 1992). *M. minimus* feeds primarily on nectar and pollen mainly from plants of the banana tree, the coconut tree and mangroves (Mickleburgh *et al*., 1992). The long- tongued nectar bat feeds by first landing on a flower and then using its long tongue to extract nectar or pollen from the flower (Mickleburgh *et al*., 1992).

2.3 Mitochondrial DNA and Cytochrome *b*

In molecular studies, there are many molecular markers exist in the mitochondrial DNA (mtDNA) such as cyt *b* and cytochrome oxidase I (COI). Mitochondrial DNA (mtDNA) is a very important molecular material in understanding the evolutionary relationship within individuals, species and populations (Irwin *et al.,* 1991). The mtDNA has been widely applied in evolutionary studies and can be used to study on population structure and gene flow, biogeography and phylogenetic relationship (Mohd Ridwan, 2010). Cyt *b* was chosen as a phylogenetic probe because it may be easier to align a protein-coding sequence that has evolved over the period spanning the origin of mammalian orders (Irwin *et al*., 1991). Cyt *b* is used to estimate the phylogenetic relationships and species limit. Based on the variation in mtDNA cyt *b* gene, the genetic values higher than 11% indicate species recognition (Baker and Bradley, 2006). Cyt *b* also have been used as a marker for the fishes and birds because it has been considered as one of the most useful genes for phylogenetic work and is probably the most known mitochondrial gene with respect to structure and function of its protein product (Pereira *et al.,* 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

A total of 55 individuals of *M. minimus* from four populations, namely Southern Sarawak (15 individuals), Central Sarawak (15 individuals), Northern Sarawak (15 individuals) and Sabah (10 individuals), were used in this study (Figure 1). Other DNA sources of *M. minimus* were retrieved from the GenBank. The examined specimens are listed in Table 1.

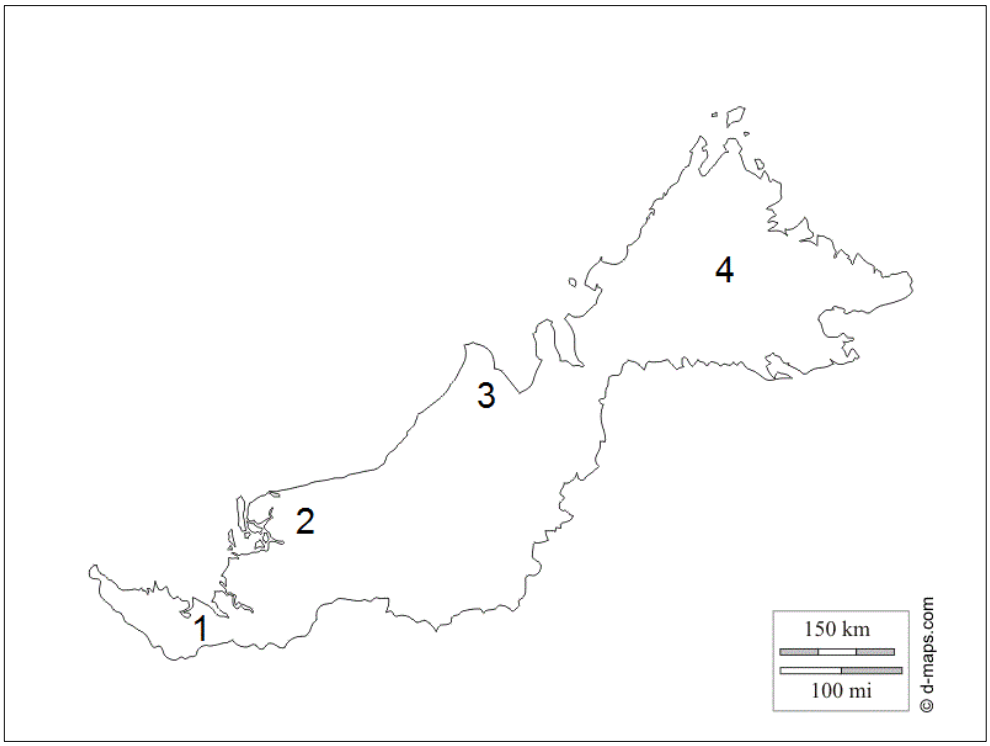

Figure 1. Map showing the type locality of *M. minimus* specimens used in molecular analyses. 1-Southen Sarawak; 2- Central Sarawak; 3- Northern Sarawak; 4- Sabah. Map modified from Dalet (2013).

Table 1. List of *M. minimus* samples used in the genetic analyses.

No.	Species	Museum No./Col. No.	GenBank Acc. No.	Locality	Populations
1	*E. major*	-	EU521600	Sarawak, Kubah National Park	-
2	*E. spelaea*	-	EU521601	Pahang, Krau Game Reserve	-
3	*M. sobrinus*	-	EU521603	Pahang, Krau Game Reserve	-
4	*M. minimus*	-	EU521602	Monggis Sub Station	Sabah
5	*M. minimus*	-	AY926645	Poring Hot Spring	Sabah
6	*M. minimus*	-	AY926646	Poring Hot Spring	Sabah
7	*M. minimus*	-	AY926647	Poring Hot Spring	Sabah
8	*M. minimus*	-	AY926648	Poring Hot Spring	Sabah
9	*M. minimus*	SRP2	-	Sebangkoi Recreational Park	Central Sarawak
10	*M. minimus*	SRP3	-	Sebangkoi Recreational Park	Central Sarawak
11	*M. minimus*	SRP4	-	Sebangkoi Recreational Park	Central Sarawak
12	*M. minimus*	SRP5	-	Sebangkoi Recreational Park	Central Sarawak
13	*M. minimus*	SRP6	-	Sebangkoi Recreational Park	Central Sarawak
14	*M. minimus*	SRP7	-	Sebangkoi Recreational Park	Central Sarawak
15	*M. minimus*	SRP9	-	Sebangkoi Recreational Park	Central Sarawak
16	*M. minimus*	MNP88	-	Mulu National Park	Northern Sarawak
17	*M. minimus*	GM796	-	Gunung Murud	Northern Sarawak
18	*M. minimus*	MNP92	-	Mulu National Park	Northern Sarawak
19	*M. minimus*	NS01023	-	Niah. Miri	Northern Sarawak
20	*M. minimus*	NS01335	-	Niah. Miri	Northern Sarawak
21	*M. minimus*	SA1	-	Sungai Asap, Belaga	Northern Sarawak
22	*M. minimus*	SS806	-	Bako National Park	Southern Sarawak
23	*M. minimus*	SS474	-	Bako National Park	Southern Sarawak

3.2 Molecular Approach

3.2.1 DNA extraction

A total of 55 samples of *M. minimus* DNA samples were successfully extracted using CTAB (cetyl-tri-metyl ammonium bromide) procedure. 700 µl 2X CTAB buffer and 5 µl of proteinase K were added into the 1 cubic millimetre minced tissue samples to denature the proteins and to eliminate the proteins which could affect the DNA product (Di Mito and Betschart, 1998). Then, 700 µl of chloroform-isoamyl alcohol were added to inhibit the lysis process. Next, the samples were centrifuged at 13000 rpm for 10 minutes and 500- 550 µl of upper layer which contain DNA were transferred into 1.5 ml fresh microcentrifuge tube. The same amount of absolute ethanol was added in the same tube. The supernatant layer was removed into a new tube containing 550 µl cold 70 % ethanol and 25 µl of NaCl, after second centrifugation takes place. The excess ethanol was discarded during third centrifugation and the pellets are allowed to dry at room temperature. The DNA was suspended in 20 µl of deionized distilled water (ddH_2O). The extracted DNA samples are kept at -80 °C in a freezer for later use.

3.2.2 DNA amplification, purification and sequencing

The partial cytochrome *b* mitochondrial DNA was used to amplify the samples of *M. minimus* (Palumbi *et al.,* 1991) (Table 2). A total volumes of 25 µl master mix was made comprising of 2.5 µl 10x buffer, 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 1.0 µl of each forward and reverse primers (10 mM), 17 µl of deionized distilled water (ddH_2O), 1.0 µl of DNA template and 0.2 µl GoTaq DNA polymerase (5 u/µl) (Table 3). The PCR was carried out using the thermocycler following to Rahman (2005) thermal profile. The amplification started with 1 minute of pre-denaturation of 94°C, followed by 30 cycles of denaturation (94°C) for 1 minute, annealing for 1 minute (56°C), extension 72°C (2 minutes), with a final extension 72°C (5 minutes) (Table 4). Amplification products were visualized using the agarose gel electrophoresis method. DNA Purification was done using the Promega Wizard SV Gel and PCR Clean Up System (Promega Co). The purified samples were then sent for sequencing at a private laboratory, 1st Base.

Table 2. Primer for cyt *b* mitochondrial used in this study and their sequences.

Primer	Sequence	Direction
GludG- L	(5'- TGACCTGAARAACCAYCGTTG- 3')	Forward
CB2H	(5'- CCCTCAGAATGATATTTGTCCTCA- 3')	Reverse

Table 3. Component of mastermix and volume used for each sample.

Component	1X Reaction (25 µl)
10x reation buffer	2.5
$MgCl_2$ (25 mM)	1.5
dNTP mix (10 mM)	0.5
Forward cyt b (10 mM)	1.0
Reverse cyt b (10 mM)	1.0
ddH_2O	17
Template DNA	1.0
Taq polymerase (5 u/µl)	0.5

Table 4. The parameter of PCR process for a total of 30 cycles.

Step	Temperature (°C)	Time (min)	Cycle
Initial denaturation	94	1	1
Denaturation	94	1	
Annealing	56	1	30
Extension	72	2	
Final extension	72	5	1
Soak	4	∞	

3.2.3 Data Analysis

CHROMAS version 1.45 (MacCarthy, 1996) was used to display the fluorescence nucleotide bases of the DNA sequence for the analysis. Multiple sequence alignments were done by using CLUSTAL X version 1.81 programmes (Thompson *et al*., 1997) and subsequently were aligned by naked eyes. Chromas version 1.45 (MacCarthy, 1996) displayed the fluorescent-based DNA sequence result that showed four types of nucleotide, namely, Adenine (A), Thymine (T), Cytosine (C) and Guanine (G). Any of noise (N) nucleotide were replaced or corrected with other nucleotide by choosing the sequence peak.

Pairwise distance between populations were computed using Molecular Evolutionary Genetic Analysis (MEGA) version 4.0 (Kumar *et al.,* 2004) with correction using a Kimura 2-parameter (K2P) model. The K2P model was used for inferring evolutionary distance in which transitions and transversions are treated separately and nucleotides were assumed to occur at the same frequency.

Phylogenetic trees were constructed using Neighbour-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods implemented in Phylogenetic Analysis using Parsimony (PAUP) version 4.0 beta (Swofford, 2001). Bayesian analysis was constructed using Mr Bayes version 3.0 (Huelsenbeck and Ronquist, 2001). The NJ clustering was performed using K2P model (Kimura, 1980). MP analysis were corresponds to Hasegawa, Kishino, and Yano 85 (HKY85) evolutionary model (Hasegawa *et al.,* 1985). Akaike Information Criterion (AIC) was used to determine the best-fit-model of sequence evolution in species by using Modeltest 3.7 (Pasoda and Crandall, 1998). Maximum Likelihood (ML) and

Bayesian tree was constructed based from evolutionary model selected by AIC. General Time Reversible (GTR) (Tavare, 1986) was used in construction of ML and Bayesian phylogenetic trees. For ML, heuristic search option was used in PAUP with Tree-bisection-reconnection (TBR) branch swapping and 10 random addition sequence replicated. Average standard deviation of the split frequencies was run until less than 0.01. The trees were rooted with three outgroups namely, *M. sobrinus*, *Eonycteris major*, and *E. spelaea*.

3.2.4 Population Genetics Analysis

The value of the haplotype (*h*) and nucleotide (π) diversities (Nei and Tajima, 1981), nucleotide divergence (Da), number of polymorphic sites (S), mean number of nucleotide differences (K) were calculated using DnaSP version 5.0 (Rozas *et al*., 2003).

The neutrality test of Tajima's D (Tajima, 1989), Fu and Li's *D** and *F** (Fu and Li, 1993) and Fu's F_s were used to test the hypothesis that all mutation are selective neutral (Kimura, 1983). Tajima *D,* is based on the differences between the number of segregation site and average number of nucleotide differences (Tajima, 1989). Although, Fu and Li's *D** and *F**, is based on molecular polymorphism data (Fu and Li, 1993). Fu's Fs (Fu, 1993), assess the haplotype structure on the haplotype frequency distribution were used as additional neutrality test. Thus, all neutrality tests were performed by DnaSP. Haplotype networks were generated using Network 4.6.1.1 (Fluxus Technology Ltd.) in order to obtain a graphical representation of the cyt *b* gene variation, minimum spanning networks (MSN).

CHAPTER 4

RESULTS

4.1 DNA Extraction

All 55 samples were successfully extracted. Several samples yield low quality products although the extraction have been done repeatedly. Some samples produced bright DNA extraction band while some are fainted and smeared. Although the samples were faint, it was enough to show that the samples contained DNA (Gani, 2011).

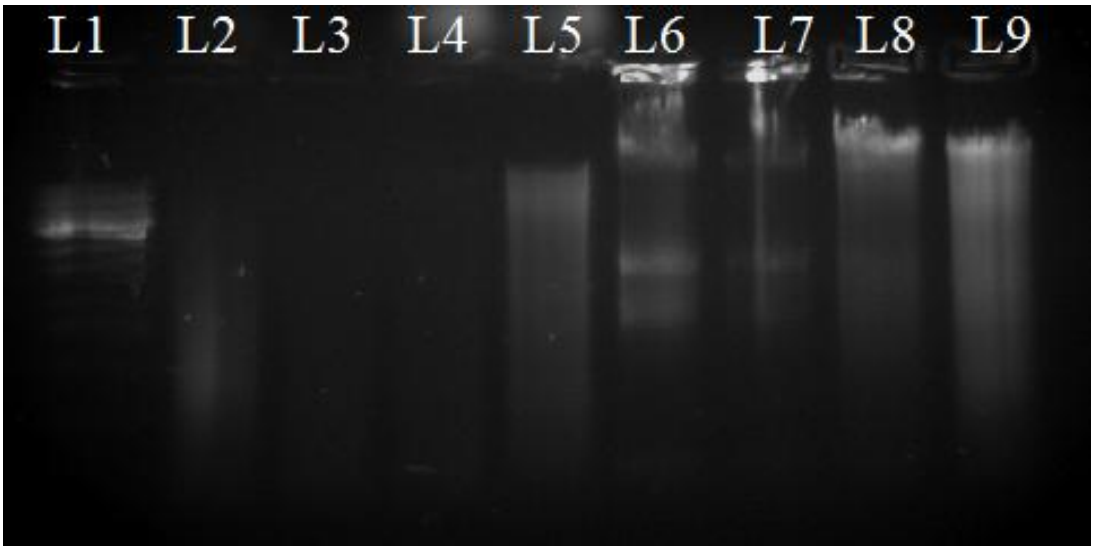

Figure 2. Gel electrophoresis of DNA extraction products.L1: Ladder 1 kb, L2: Sample SRP1, L3: SRP2 and L4: SRP3 was negative, without any band yield. L5: Sample SRP4, L6: SRP5, L7: SRP6, L8:SRP7 and L9: SRP9 showed bright extracted DNA bands.

4.2 PCR and Purification

Samples that were successfully extracted are amplified by followed the PCR profile of *M. minimus*. However, not all extraction samples were successfully amplified. Out of 55 samples, only 27 were successfully amplified. All positive PCR products were purified using Fermentas Purified Kit before sending for DNA sequencing.

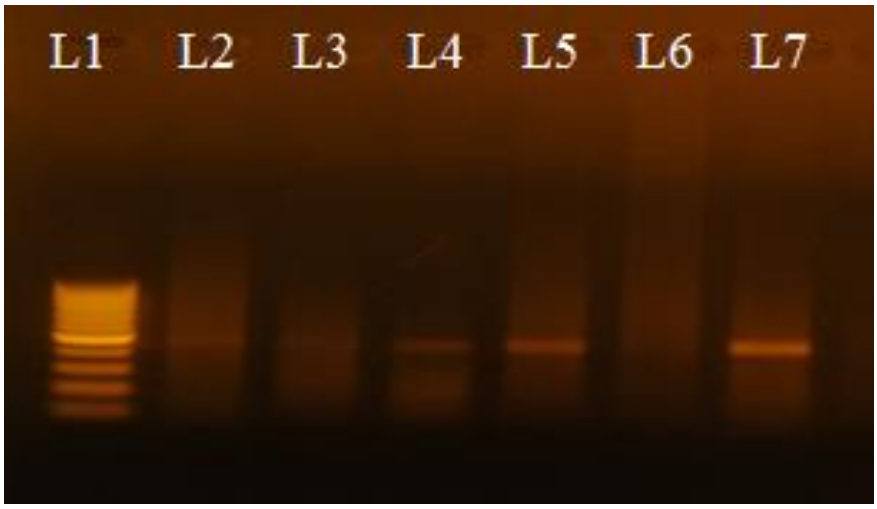

Figure 3. Gel electrophoresis of PCR products with 1% of agarose gel. L1: DNA ladder (100bp). L2: SRP2. L 3: SRP3. L 4: SRP4. L5: SRP5. L 6: SRP6. L7: SRP 7

4.3 Sequencing and Alignment

Out of 27 samples, only 15 samples were successfully sequenced. The rest of samples are not reliable for tree construction due to low peak of nucleotide and short length of sequence.

4.4 Sequence Variation Analysis and Nucleotide Composition

Based on the 377 bp of partial cyt *b*, there are 22 characters (5.84%) were conserved sites, 355 characters (94.15%) were variable site and 287 characters (76.15%) were parsimoniously informative sites. Furthermore, singletons are 97.61% which made up of 368 characters. For the nucleotide composition, the highest percentage of average nucleotide composition among all individuals is Adenine (A) which is 28.3 %, followed by Thymine (T) 27.9 %, Cytosine (C) 26.8 % and Guanine (G) 16.9 %. Table 5 below showed the percentages of nucleotide frequencies from 23 individuals in respect to outgroups, *M. sobrinus*, *Eonycteris spelaea* and *E. major*. Adenine has the highest percentage of nucleotide frequencies ranging from 31.3 % to 17 % while Guanine has the lowest percentage of nucleotide frequencies ranging from 14.6 % to 23.3 %.

Table 5. Percentage of nucleotide composition among 23 individuals of *M. minimus* together with outgroups.

No	Samples	T	C	A	G
1	*M. minimus* (Sabah)AY926645	29.7	25.5	30.2	14.6
2	*M. minimus* (Sabah) AY926648	29.7	25.5	30.2	14.6
3	*M. minimus* (Sabah) EU521602	29.7	25.5	30.2	14.6
4	*M. minimus* (Sabah) AY926646	30.0	25.2	30.2	14.6
5	*M. minimus* (Sabah) AY926647	29.4	25.7	30.2	14.6
6	*M. sobrinus* (Pahang) EU521603	29.7	25.7	30.0	14.6
7	*M. minimus* (Central Sar.) SRP5	29.4	25.7	30.2	14.6
8	*M. minimus* (Central Sar.) SRP4	28.6	26.0	30.5	14.9
9	*M. minimus* (Central Sar.) SRP7	28.9	25.7	30.5	14.9
10	*M. minimus* (Northern Sar.) SA1	29.2	25.7	30.5	14.6
11	*M. minimus* (Central Sar.) SRP6	28.1	24.4	31.3	16.2
12	*M. minimus* (Northern Sar.) NS01335	28.4	24.7	31.0	15.9
13	*M. minimus* (Central Sar.) SRP2	24.7	24.1	27.9	23.3
14	*M. minimus* (Northern Sar.) NS01023	25.2	23.3	31.8	19.6
15	*M. minimus* (Central Sar.) SRP3	26.5	23.6	31.0	18.8
16	*M. minimus* (Central Sar.) SRP9	27.6	23.6	30.5	18.3
17	*M. minimus* (Northern Sar.) MNP092	31.3	24.1	27.3	17.2
18	*E. major* EU521600	27.3	29.4	26.8	16.4
19	*E. spelaea* EU521601	26.5	29.4	27.6	16.4
20	*M. minimus* (Northern Sar.) MNP88	24.1	34.0	21.0	21.0
21	*M. minimus* (Northern Sar.) GM796	25.2	36.9	17.0	21.0
22	*M. minimus* (Southern Sar.) SS806	26.3	26.5	26.0	21.2
23	*M. minimus* (Southern Sar.) SS474	26.5	36.6	19.6	17.2
	Average	27.9	26.8	28.3	16.9